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14. ABSTRACT
Genetic analyses on prostate cancer has been occurring for over a decade. However, such studies were uniformly performed on the entire tumor without regard to its components despite the fact that a few groups were quite aware of both epithelial and stromal components of tumors, and the cell biology of the tumor "microenvironment" has been described for the last 20 years. Thus, until now, when a genetic alteration, be it intragenic mutation, regional amplification, or deletion manifested by loss of heterozygosity of markers (LOH) is attributed to a prostate cancer, it is unclear if the alteration is actually occurring in the epithelial compartment, the surrounding stromal compartment or both. Our own preliminary data on breast carcinomas demonstrate that LOH and even somatic mutations can occur in surrounding stromal fibroblasts. Therefore, this proposal proposes to search for genetic alterations in the stroma of prostate cancers and to determine if such alterations can influence clinical outcome. In the first year, we have accrued 55 distinct, non-M1, sporadic adenocarcinomas of the prostate, have subjected them to laser capture microdissection to separate compartments, and are about to begin a total genome LOH scan.

15. SUBJECT TERMS
human cancer genetics, prostate cancer stroma

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Final Report

Proposal Title: Genetic alterations in epithelial and stromal compartments of prostate adenocarcinomas (DAMD-02-1-0118)
PI: Charis Eng, MD, PhD

INTRODUCTION

Prostate cancer is common in the West and is uniformly lethal once metastasized. Thus, there is growing interest in examining the genetic alterations in prostate cancer. Until recently, however, solid tumors such as prostate carcinoma was treated as a single amorphous entity. Genetic studies were uniformly performed on the entire tumor without regard to its components despite the fact that a few groups were quite aware of both epithelial and stromal components of tumors, and the cell biology of the tumor "microenvironment" has been described for the last 20 years. Thus, until now, when a genetic alteration, be it intragenic mutation, regional amplification, or deletion manifested by loss of heterozygosity of markers (LOH) is attributed to a prostate cancer, it is unclear if the alteration is actually occurring in the epithelial compartment, the surrounding stromal compartment or both. Recently, Moinfar and colleagues, using a limited subset of samples and markers, demonstrated that LOH of markers representing three chromosomal loci can occur in the stromal compartment of a small pilot series of invasive breast adenocarcinomas (1). Further, the PI has demonstrated LOH of a limited set of markers in the stroma of invasive breast adenocarcinomas (2). More importantly, somatic intragenic mutations of *TP53* and *PTEN* have been found in the stroma, but are mutually exclusive within any single compartment (3). This has never been examined in prostate cancers. Nonetheless, the mechanisms, especially the genetic mechanisms, by which the different cells in the micro-environment interact with the epithelial component to initiate and/or promote tumor growth is not well understood. Thus, the overall hypothesis of the submitted proposal was that genetic changes in the stromal and epithelial compartment of prostate adenocarcinomas differentially contribute to tumor growth, such that they affect clinical outcomes differently. The hypothesis is to be addressed by two Objectives:

1. To determine the relative frequency of genetic alterations within the stromal versus epithelial compartment of human prostate adenocarcinomas and to build a genetic model for multistage stepwise carcinogenesis involving the epithelium and stroma in prostate cancer;

2. To determine the clinical consequences of genetic alterations within the stromal versus epithelial compartment of adenocarcinomas of the prostate.

BODY

Objective 1: To determine the relative frequency of genetic alterations within the stromal versus epithelial compartment of human prostate adenocarcinomas, and to build a genetic model for multistage stepwise carcinogenesis involving the epithelium and stroma in prostate cancer

To characterize global genomic alterations in prostate cancer stroma and epithelium, 381-microsatellite LOH/AI genome scan of DNA derived from LCM-captured epithelium and stroma of 116 $T_{any}N_{any}M_0$ sporadic prostate cancers. 371 markers across all chromosomes, ranging from

7 on chromosome 22 to 31 on chromosome 1, were analyzed. 38,460 PCR-reactions (19,639 for epithelium, 18,821 stroma) were informative for LOH/AI evaluation. 20,188 (52.5%) LOH/AI events [9,742 (49.6%) in epithelium, 10,446 (55.5%) stroma] occurred. Average LOH/AI frequencies over entire chromosomes ranged from 42% to 58% in epithelium and 51% to 69% in stroma.

Per chromosome, overall average LOH/AI frequency in the stroma was uniformly found to be higher than that in the epithelium, in contrast to breast cancer (4). A marginal model was used to compare LOH/AI frequencies between epithelium and stroma for each chromosome, yielding model-based estimates for the LOH/AI frequencies and a p-value for the comparison. Stromal LOH/AI frequencies were significantly higher than those in epithelium for 16 of the chromosomes at 0.05 significance level and for 13 chromosomes (1, 2, 3, 4, 5, 6, 11, 15, 16, 18, 19, 20, 22) after adjustment for multiple testing by using 0.05/23 ($P \leq 0.002$) as the significance level. Chromosome-wise average LOH/AI frequencies in 13 chromosomes are higher in the stroma than in the epithelium. Model-based statistics revealed 16 markers in epithelium and 8 in stroma with significantly higher LOH/AI frequencies compared to other markers on the same chromosome. Informatics analysis and formal statistics indicated that the LOH/AI profile of epithelium and stroma from a single subject tended to be more similar than samples from different subjects, again, in contrast to sporadic breast cancers (4, 5).

For details, please see prepared manuscript in Appendix.

Objective 2: To determine the clinical consequences of genetic alterations within the stromal versus epithelial compartment of adenocarcinomas of the prostate

By studying the distributions and correlations between compartment-specific genomic alterations and the 3 CPF, tumor size, tumor grade and regional lymph node (LN) status, we found tumor size and grade to be closely (positively) related. Samples with metastases to regional LN also tend to have higher grade and large size of tumors, but the number of such samples is small.

Hierarchical Clustering and Multi-dimensional Scaling of Compartment-Specific LOH/AI Profiles with Respect to CPF

To study the relationship between CPF at presentation and the overall LOH/AI pattern, 116 samples were clustered by combining the epithelial and stromal samples from each of the same subjects. In effect, each subject is considered to have one sample, with 371x2=742 distinct markers. We also performed multi-dimensional scaling combining epithelial and stromal samples of the same subject.

In another clustering analysis, only the markers found to have $p \leq 0.05$ in the hotspot analysis with at least 5 informative cases were included. We focused on these important markers in order to reduce any potential noise in the data. Due to the strong evidence of similar LOH/AI between epithelium and stroma noted above, when a marker is included, both LOH/AI in the epithelium and in the stroma for this marker is used in the clustering.

By labeling information for each of the CPF in the clustering results above, we see evidence of a relationship between the overall LOH/AI pattern and tumor grade. To formally test these associations, we divide the samples based on the first branching in the hierarchical clustering and compare the two groups of samples by performing the rank sum test for tumor grade and tumor size and Fisher's exact test for LN status. The p-values for the clustering based on all markers (and clustering based on hotspots only) are tumor grade P=0.002 (0.003); tumor size P=0.028 (0.016); and LN status P=0.16 (0.041). Thus, we see highly significant

associations between overall LOH/AI patterns and tumor grade, in particular. The significant association with tumor size may be due to the inter-dependent relationship between tumor size and tumor grade. The results also suggest that markers that are hotspots may be more relevant to CPF than merely all the other markers, and so, considering only these markers reduces the noise in the data, especially for LN status.

Association between Compartment-Specific LOH/AI and CPF

The average LOH/AI frequency across each chromosome, in carcinomatous epithelium and stroma, respectively, was then tested for its association with CPF. The chromosome-wise LOH frequencies were used because they carry more information than those for single markers. After adjusting for multiple testing to control for the overall type I error rate at 5%, we found that the only significant results are for the relationship between tumor grade (a binary variable indicating grade of 7 or higher) and LOH/AI at 6 chromosomes in epithelium (chromosomes 4, 5, 8, 14, 16, 18) and at 1 chromosome in stroma (chromosome 4). It should be noted that the association is significant for a number of additional chromosomes at the 0.05 level, and the results in the epithelium and the stroma are similar for many chromosomes.

To confirm the association between chromosome-wise LOH/AI frequencies and tumor grade, we performed further analysis based on logistic regression. Given the candidate chromosomes above, a stepwise model building procedure was used to construct a model, based on which the predicted probabilities of having a higher grade were calculated for all samples. The average predicted probabilities follow a consistent pattern over the entire range of actual tumor grade, although only the binary information of having a grade of at least 7 has been used in the model fitting. This strengthens the reliability of our finding.

KEY RESEARCH ACCOMPLISHMENTS

This is the first report investigating whole genome LOH/AI in prostate cancer stroma, ie, the prostate tumor microenvironment, and the first correlating global compartment-specific genomic alterations with CPF. Importantly, we have demonstrated that specific regional alterations on specific chromosomes in the stroma occur and that these are correlated with Gleason grade, but not other CPF's.

REPORTABLE OUTCOMES

Promoted to Professor of Medicine, Human Cancer Genetics and Molecular Genetics, The Ohio State University, Jul. 1, 2002.

Promoted to Director, Division of Human Genetics, Department of Internal Medicine, The Ohio State University, Oct. 1, 2002.

Conferred the Dorothy E. Klotz Endowed Chair in Cancer Research by The Ohio State University, Dec. 1, 2002.

Doris Duke Distinguished Clinical Scientist Award, 2002-

Elected Fellow, AAAS, Sept., 2003

Chair, Scientific Program Committee, 53rd Annual Meeting of the American Society of Human Genetics, Los Angeles, CA, Nov. 4-8, 2003

Appointed Senior Editor, *Cancer Research*, Jan. 1, 2004 –

Elected Member, Association of American Physicians (AAP), April, 2004

Appointed Member, American Association for Cancer Research (AACR) Publications Committee, April, 2004 –

Donald Unverfurth Award for Outstanding Research Achievements and Mentorship in the Department of Internal Medicine, The Ohio State University, Columbus, 2005

Appointed Chair and Founding Director, Genomic Medicine Institute, Cleveland Clinic Foundation, Cleveland, OH, Sept, 2005-

Appointed Professor and Vice Chairman, Department of Genetics, Case Western Reserve University School of Medicine, Cleveland, OH, Sept, 2005-

ATA Van Meter Award for Excellence in Research on the Thyroid and Related Organs 2005

CONCLUSIONS

This is a paradigm-shifting work that demonstrates the monoclonal genomic alterations do occur in tumor stroma of sporadic prostate carcinomas. This is not a generalized genomic instability as very specific chromosomal regions are altered in the stroma. Of note, somatic alterations in both the epithelium and stroma contribute to tumor aggressiveness.

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APPENDIX

Manuscript in Preparation: Sawada T, Shen L, Zheng P, Eng C. Microenvironment-Profiling of Prostate Cancer Reveals Genomic Instability of Chromosome 4 in the Stroma and of Six Chromosomes in the Epithelium Associated with Tumor Grade.

Title: Microenvironment-Profiling of Prostate Cancer Reveals Genomic Instability of Chromosome 4 in the Stroma and of Six Chromosomes in the Epithelium Associated with Tumor Grade

Running Title: Microenvironment-Profiling of Prostate Cancer

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ABSTRACT

Background Recent studies suggest the importance of tumor microenvironment in carcinogenesis. The role of global loss of heterozygosity (LOH)/allelic imbalance (AI) in neoplastic epithelium and tumor stroma and how they interact in prostate carcinogenesis to affect clinical features is unknown.

Objective To characterize global genomic alterations in prostate cancer stroma and epithelium and their association with clinico-pathological factors.

Design, Setting, and Patients 381-microsatellite LOH/AI genome scan of DNA derived from epithelium and stroma of 116 $T_{any}N_{any}M_0$ sporadic prostate cancers.

Main Outcome Measures Frequencies of overall LOH/AI and hotspots in epithelial and stromal compartments. Stromal and epithelial LOH/AI profiles assessed for associations with presence of regional lymph node metastasis, tumor grade, and tumor size.

Results Chromosome-wise average LOH/AI frequencies in 13 chromosomes are higher in the stroma than in the epithelium. Model-based statistics revealed 16 markers in epithelium and 8 in stroma with significantly higher LOH/AI frequencies compared to other markers on the same chromosome. Informatics analysis and formal statistics indicated that the LOH/AI profile of epithelium and stroma from a single subject tended to be more similar than samples from different subjects. LOH/AI for 6 chromosomes in the epithelium and 1 in the stroma were associated with tumor grade.

Conclusion In prostate cancers, the total genome LOH/AI profile is similar in both compartments from the same subject, suggestive of the presence of tumor-microenvironment interaction possibly induced by EMT. Compartment- and chromosome-specific genomic

instability correlates significantly with tumor grade, suggesting new biomarkers of prognosis and targets for therapy.

INTRODUCTION

Prostate cancer is one of the leading causes of cancer and cancer-related deaths among men in the United States and Western Europe.(6) Recent investigation has highlighted the tumor microenvironment in cancer progression. Genetic changes in tumor stroma have been reported for a few types of solid tumors,(1-3, 7-11) although only selected markers have been used in most of these studies assessing loss-of-heterozygosity (LOH)/allelic imbalance (AI).

In prostate cancer (CAP), it has been suggested that tumor microenvironment must play an important role from progression, including acquisition of androgen independence, to distant metastases.(12, 13) It has also been suggested that tumor-microenvironment interactions through diffusible soluble factors, such as TGF-beta, as well as the extracellular matrix (ECM) leads to the development of metastasis.(13) Furthermore, the possibility of epithelial-mesenchymal transition (EMT) has been proposed,(13) although there is controversy about this mechanism in cancer progression.(14, 15) Nonetheless, in CAP, little is known about the genetic basis of the microenvironment: only one report described genotypic heterogeneity in mesenchymal cells with a small number of markers.(11) Still, this suggested that genetic alterations in CAP stroma exist and may be biologically relevant.

Recent studies have suggested that carcinoma and adjacent stroma are all derived from a common progenitor cell.(8, 10) In contrast, however, we have found that the spectrum of genomic alterations is distinct in sporadic breast cancer and likely independent between the stroma and neoplastic epithelium, suggesting that both elements are clearly not derived from a common progenitor clone but rather undergo similar selective pressures, whether in the epithelial carcinoma or in the tumor microenvironment.(4) To clarify whether global genomic alterations do occur in the tumor stroma and epithelium in prostate carcinoma and to see if there is a

different spectrum of LOH/AI from sporadic breast cancer, we performed a whole genome LOH/AI scan using 381-microsatellite markers. Importantly, we also sought to determine if compartment-specific LOH/AI can be correlated with presenting clinico-pathologic features (CPF).

MATERIALS AND METHODS

Patients

116 $T_{any}N_{any}M_0$ prostate carcinomas were obtained and analyzed in accordance with the respective institution's Human Subjects Protection Committees. All slides were re-reviewed by a single genito-urinary pathologist (PZ). Clinical information such as the presence of lymph node metastasis, tumor grade (Gleason score), and tumor size were noted (Table 1S).

Laser Capture Microdissection (LCM) and Total Genome LOH/AI Scan

LCM was performed using the Arcturus PixCell II microscope (Arcturus Engineering Inc., Mountain View, CA) to isolate neoplastic epithelium and tumor stroma separately.(2-4, 7, 16) We captured stromal fibroblasts adjacent to malignant epithelium the tumor stroma) under direct microscopic observation.(2-4, 7, 16) Stromal fibroblasts resided in between aggregations of epithelial tumor cells or no more than 0.5 cm distant from a tumor nodule. LCM is able to control for proximity of stroma to carcinoma cells amongst all samples. While epithelial-stromal cell cross-contamination is a possibility, we utilized standard and well-worked out protocols to minimize this, as reported previously.(3, 4, 16, 17) Corresponding normal DNA for each case was procured from normal tissue, obtained a large distance from the tumor site or from a

different tissue block containing only normal tissue (latter first choice). The different origins of the corresponding normal DNA had no effect on the frequency or pattern of LOH/AI.

Genomic DNA was extracted as previously described, with the exception that incubation in proteinase K was done at 65 C for two days.(2) Primer sets for multiplex PCR defined 381 microsatellite markers in 72 multiplex panels (Research Genetics, Invitrogen). Genotyping, analyses, and scoring of LOH/AI were done as reported.(4, 15, 18) The methodological veracity of LOH/AI using multiplex-PCR on archived templates was extensively validated as published.(4, 16)

Data Analysis

The dataset contains LOH status at 381 markers for 116 different samples, each with neoplastic epithelium, tumor stroma and normal tissue. Ten markers were never informative in at least one compartments, and excluded. Two types of marginal models were used, the first to estimate chromosome-wise average LOH probabilities and the second to detect elevated LOH frequency at a given marker. In the former, one model was fitted for each chromosome with only one regression coefficient (the intercept). In the latter, one model was fitted for each marker to data from the same chromosome, with the only term being the indicator for this marker and the significance of its coefficient tested using a Wald test. That is, the LOH frequency at this marker was compared to the average LOH frequency over the rest of the same chromosome. In all models, the compound symmetry working correlation structure was used. To account for a number of features of LOH/AI data such intra-sample correlations and informativeness that differs among markers and among samples, marginal models for correlated data and the GEE estimation method of Diggle et al.(19) were used, yielding more efficient inference than simple

average LOH/AI frequencies, and without strong parametric assumptions. For each chromosome in each compartment, model-based estimates of marker-wise LOH/AI probabilities were obtained from fitting a marginal model that allows the LOH/AI probability of each marker to be distinct. Furthermore, to detect markers with elevated LOH/AI frequencies, for each marker in each compartment, a marginal model was fitted to compare the LOH/AI probability of this marker with the average LOH/AI probability of the rest of the chromosome. To adjust for multiple comparisons and control the overall alpha level for each compartment to 0.05, the Bonferroni method was used so that the alpha value used in a single comparison is 0.05 divided by the total number of comparisons made. Similarities between samples in terms of LOH/AI events were studied using hierarchical clustering and multi-dimensional scaling, with the dissimilarity between each pair of samples measured by the proportion of discordant LOH/AI, ie, the proportion of markers being LOH/AI in one sample and ROH in the other among all markers that are informative in both samples. Average linkage was used in hierarchical clustering. The clustering results were related to each clinical variable, both graphically and via formal tests, Wilcoxon rank sum test and Fisher's exact test, adjusting for multiple testing (Bonferroni method). A stepwise model building process for logistic regression was undertaken to confirm and further explore the relationships found to be statistically significant. All data analysis was done with the statistical package R, version 1.8.1.

RESULTS

Overall Marker- and Chromosome-wise LOH/AI Frequencies

371 markers across all chromosomes, ranging from 7 on chromosome 22 to 31 on chromosome 1, were analyzed. 38,460 PCR-reactions (19,639 for epithelium, 18,821 stroma) were

informative for LOH/AI evaluation. 20,188 (52.5%) LOH/AI events [9,742 (49.6%) in epithelium, 10,446 (55.5%) stroma] occurred. Average LOH/AI frequencies over entire chromosomes ranged from 42% to 58% in epithelium and 51% to 69% in stroma.

Per chromosome, overall average LOH/AI frequency in the stroma was uniformly found to be higher than that in the epithelium (Fig. 1a), in contrast to breast cancer.(4) A marginal model was used to compare LOH/AI frequencies between epithelium and stroma for each chromosome, yielding model-based estimates for the LOH/AI frequencies and a p-value for the comparison. Stromal LOH/AI frequencies were significantly higher than those in epithelium for 16 of the chromosomes at 0.05 significance level and for 13 chromosomes (1, 2, 3, 4, 5, 6, 11, 15, 16, 18, 19, 20, 22) after adjustment for multiple testing by using 0.05/23 ($P \leq 0.002$) as the significance level (Fig. 1b, Table 2).

Hierarchical Clustering and Multi-dimensional Scaling of LOH/AI Profile of CAP Epithelium Compared to its Stroma

We then sought to determine whether the tumor microenvironment LOH/AI profiles were more similar to one another or whether each stromal LOH/AI profile is more similar to that of its corresponding carcinomatous epithelium. The dissimilarity between each pair of samples can be measured by the proportion of discordant LOH/AI, ie, the proportion of markers being LOH/AI in one sample and ROH in the other among all markers that are informative in both samples. Hierarchical clustering and multi-dimensional scaling of all 232 samples (116 epithelial, 116 stromal) do not reveal any overall closeness amongst the profiles of the epithelial samples alone, nor for the profiles of the stromal samples alone. In contrast, LOH/AI profiles of the epithelium and corresponding stroma from the same subjects tend to cluster closely together (Fig. 2). This

closeness can be visualized by connecting each of such pairs of samples in the multi-dimensional scaling plot (Fig. 2), in which the distance between two samples closely approximates their dissimilarity measure.

We then numerically studied the closeness of each corresponding epithelial and stromal sample pairs by comparing the percentages of discordant LOH/AI between paired epithelial-stromal pairs compared to those between other (non-corresponding) pairs of samples (Fig. 3). The former have a mean of 38.5% (SD7.3%) whereas the latter has a mean 47.4% (SD 10.6%). To formally test the hypothesis that epithelial and stromal samples from the same subject exhibit similar overall LOH/AI profiles, we compared the degree of similarity between paired epithelium/stroma samples with their similarities to other samples. Specifically, for each epithelium (or stroma) sample, we rank its numerical dissimilarity score to the corresponding stroma (or epithelium) sample relative to its dissimilarities to all other 231 samples. A rank of 1 indicates that its corresponding stroma is closer to its epithelium than to any other sample. All 232 ranks together (median=36.5), one for each sample, were then compared to their expected median value of 231/2=115.5 under the null hypothesis (i.e. paired epithelium and stroma samples are overall no more similar than other pairs of samples) using the non-parametric signed rank test. The null hypothesis was rejected at $p= 3.4 \times 10^{-34}$, indicating very strong evidence for similarity between paired epithelium and its corresponding stroma, in contrast to sporadic breast cancer samples.(4)

Markers with Compartment-Specific Elevated LOH/AI Frequencies in CAP and Comparison with Sporadic Breast Cancer

A marker locus with elevated LOH/AI frequency compared to other markers on the same chromosome suggests a potential nearby tumor suppressor gene. When the frequency of LOH/AI at a marker is significantly higher than that of other markers along the same chromosome, the is operationally termed a "hotspot".(4) A statistical model that accounts for intra-sample correlations is used to test, for each marker, whether its marginal (averaged over the entire population of patients) LOH/AI frequencies are significantly higher than those of the other markers on the same chromosome (after averaging). This exercise was performed for the epithelium and stroma separately. To adjust for multiple testing, the Bonferroni method is applied that uses 0.05/371=0.00013 (instead of 0.05) as the significance level. To further reduce the chance of false positives, we also required the number of informative cases to be at least 14 to ensure that uniform (100%) LOH/AI purely by chance is extremely unlikely (probability less than 0.05/371=0.00013). We found 16 LOH/AI hotspots in the epithelium (Table 3a) and 8 in the stroma amongst our CAP samples (Table 3b). A simpler method by Miller et al.(18) was also used for these same comparisons, and the result is also significant ($p<0.00013$ [0.05/371]) for all 24 hotspots, thus strengthening our observations. Interestingly, 12 of 16 hotspots in epithelium also had significantly elevated LOH/AI frequencies in the stroma at the 0.003 [0.05/16] level, and 6 of 8 hotspots in stroma have significantly elevated LOH/AI frequencies in the epithelium ($p=0.0063$ [0.05/8]). The probabilities of these happening by chance, based on the hypergeometric distribution, are 2.3×10^{-9} and 1.6×10^{-4}, respectively. Therefore, our data suggest that the similarity of genome-wide LOH/AI profiles between each sample's carcinomatous epithelium and its corresponding stroma is, to a very high probability.

To compare the LOH/AI tumor stroma profiles between CAP and those of breast cancer samples, we looked at 57 marker loci previously found to be hotspots for sporadic breast cancer

samples.(4) Of these 57 markers, 55 are among the 371 in the current study. It is noteworthy that 67.3% of these markers have p-values that are less than 0.05, a proportion significantly higher than the 30.7% of all the markers in the current study with such small p-values (Table 4). We then utilized Fisher's exact test to test the null hypothesis that these 55 previously found hotspots for sporadic breast cancers (epithelium and/or stroma) constitute a random sample of the 371 markers in terms of their evidence for hotspots, ie, that these are found by coincidence or chance. We were able to reject the null hypothesis by finding a very strong indication ($p=7.4 \times 10^{-9}$) that sporadic prostate and breast cancers share some common LOH/AI hotspots, which suggests the existence of important chromosomal regions common to the pathogenesis of at least these 2 types of solid tumors.

Clinico-Pathological Features (CPF)

By studying the distributions and correlations between compartment-specific genomic alterations and the 3 CPF, tumor size, tumor grade and regional lymph node (LN) status, we found tumor size and grade to be closely (positively) related. Samples with metastases to regional LN also tend to have higher grade and large size of tumors, but the number of such samples is small.

Hierarchical Clustering and Multi-dimensional Scaling of Compartment-Specific LOH/AI Profiles with Respect to CPF

To study the relationship between CPF at presentation and the overall LOH/AI pattern, 116 samples were clustered by combining the epithelial and stromal samples from each of the same subjects (Fig. 4). In effect, each subject is considered to have one sample, with 371x2=742

distinct markers. We also performed multi-dimensional scaling combining epithelial and stromal samples of the same subject.

In another clustering analysis, only the markers found to have $p \leq 0.05$ in the hotspot analysis with at least 5 informative cases were included (Fig. 5). We focused on these important markers in order to reduce any potential noise in the data. Due to the strong evidence of similar LOH/AI between epithelium and stroma noted above, when a marker is included, both LOH/AI in the epithelium and in the stroma for this marker is used in the clustering.

By labeling information for each of the CPF in the clustering results above, we see evidence of a relationship between the overall LOH/AI pattern and tumor grade. To formally test these associations, we divide the samples based on the first branching in the hierarchical clustering and compare the two groups of samples by performing the rank sum test for tumor grade and tumor size and Fisher's exact test for LN status. The p-values for the clustering based on all markers (and clustering based on hotspots only) are tumor grade P=0.002 (0.003); tumor size P=0.028 (0.016); and LN status P=0.16 (0.041). Thus, we see highly significant associations between overall LOH/AI patterns and tumor grade, in particular. The significant association with tumor size may be due to the inter-dependent relationship between tumor size and tumor grade. The results also suggest that markers that are hotspots may be more relevant to CPF than merely all the other markers, and so, considering only these markers reduces the noise in the data, especially for LN status.

Association between Compartment-Specific LOH/AI and CPF

The average LOH/AI frequency across each chromosome, in carcinomatous epithelium and stroma, respectively, was then tested for its association with CPF. The chromosome-wise LOH

frequencies were used because they carry more information than those for single markers. After adjusting for multiple testing to control for the overall type I error rate at 5%, we found that the only significant results are for the relationship between tumor grade (a binary variable indicating grade of 7 or higher) and LOH/AI at 6 chromosomes in epithelium (chromosomes 4, 5, 8, 14, 16, 18) and at 1 chromosome in stroma (chromosome 4). It should be noted that the association is significant for a number of additional chromosomes at the 0.05 level, and the results in the epithelium and the stroma are similar for many chromosomes (Table 5).

To confirm the association between chromosome-wise LOH/AI frequencies and tumor grade, we performed further analysis based on logistic regression. Given the candidate chromosomes above, a stepwise model building procedure was used to construct a model, based on which the predicted probabilities of having a higher grade were calculated for all samples. The average predicted probabilities follow a consistent pattern (Fig.6) over the entire range of actual tumor grade, although only the binary information of having a grade of at least 7 has been used in the model fitting. This strengthens the reliability of our finding.

DISCUSSION

Recent molecular studies have provided a myriad of information about genetic changes in prostate cancer. In epithelium, several candidate tumor suppressor genes in LOH/AI regions include *NKX3.1* (located at 8p21), *PTEN* (10q23), *CDKN1B* (12p12), *RB* (13q14), and *TP53* (17p13).(6, 20, 21) This is the first report investigating whole genome LOH/AI in prostate cancer stroma, ie, the prostate tumor microenvironment, and the first correlating global compartment-specific genomic alterations with CPF. Clustering methods and tests based on discordant LOH/AI percentages provide strong evidence for microenvironmental input in

prostate cancer progression, possibly induced by EMT where one would expect the epithelial LOH/AI profile would be more in common with its corresponding stroma than any other sample. That LOH/AI hotspots are similar in both compartments might suggest the presence of genes involved in EMT and/or a common progenitor stem cell which evolves into both carcinomatous epithelium and surrounding stroma. The most radical postulate, not inconsistent with our data, would be that it is the tumor microenvironment that undergoes the first genomic alterations. This is most likely true in breast cancers from individuals with *BRCA1/2* germline mutation.(16) While this is plausible in breast cancers associated with initiating germline *BRCA1/2* mutations, it is a little difficult to understand in the sporadic prostate carcinogenesis context.

In comparison with the LOH/AI spectra in the epithelium and stroma in sporadic breast cancer,(4) it is noteworthy that there are some common LOH/AI hotspots between breast and prostate cancers, reflecting the importance of these markers for solid tumor initiation or progression. Further, it is interesting that LOH/AI is more frequent in the stroma than in the epithelium in prostate cancer, whereas the average LOH/AI frequencies for all chromosomes at the individual marker level is higher in the epithelium than in the stroma in breast cancer. This difference may be due to the degree of EMT and/or a common stem cell progenitor which already possesses LOH/AI which develops into both the carcinomatous epithelum as well as the tumor stromal cells.

In conclusion, we have shown that overall LOH/AI is more frequent in stroma than epithelium in prostate cancers, and that LOH/AI profiles in the epithelium and stroma of each of the subjects cluster closely together, reflecting the importance of the microenvironment in initiation and progression. Finally, we demonstrated that overall genomic instability of chromosome 4 in the stroma and of 6 chromosomes in the neoplastic epithelium are associated

with tumor grade, and by association, tumor size. This observation provides fundamental insight into chromosome-specific, compartment-specific roles in prostate carcinogenesis. Our data may also reveal the solid tumor microenvironment as a novel compartment for important biomarkers as well as targeted therapy.

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Table 1S. Clinical information of 116 subjects in this study [**For Supplement Only**]

Gleason grade	_
≤ 6	73 (63)
7 to 9	39 (34)
N/A	4 (3)
_	_
Lymph node metastasis	_
positive	10 (9)
negative	101 (87)
N/A	5 (4)
_	_
Tumor size	_
<=2 cm	58 (50)
>2 cm	54 (47)
N/A	4 (3)

Table 2. Observed and estimated (model-based) LOH frequencies in the epithelium and the stroma for each chromosome [**For Supplement Only**]

Chromosome	Simple average LOH frequency in the epithelium	Simple average LOH frequency in the stroma	Model-based LOH frequency in the epithelium	Model-based LOH frequency in the stroma	p-value for equal LOH frequency in the epitheilum and stroma
1	0.475	0.534	0.487	0.552	0.00085
2	0.475	0.53	0.483	0.54	0.0015
3	0.488	0.569	0.503	0.586	2.6E-05
4	0.501	0.582	0.516	0.602	0.00015
5	0.525	0.596	0.544	0.62	0.0018
6	0.546	0.622	0.555	0.633	0.00052
7	0.539	0.58	0.548	0.592	0.043
8	0.534	0.555	0.543	0.563	0.27
9	0.549	0.57	0.56	0.58	0.37
10	0.543	0.586	0.559	0.605	0.052
11	0.426	0.544	0.435	0.553	4.0E-06
12	0.49	0.512	0.495	0.52	0.22
13	0.512	0.516	0.514	0.517	0.90
14	0.436	0.514	0.442	0.518	0.0051
15	0.518	0.592	0.534	0.61	0.00051
16	0.489	0.553	0.499	0.564	0.00043
17	0.526	0.533	0.536	0.544	0.76
18	0.451	0.536	0.479	0.564	0.0018
19	0.449	0.549	0.466	0.57	0.00048
20	0.418	0.533	0.427	0.548	7.3E-06
21	0.526	0.54	0.526	0.541	0.64
22	0.419	0.513	0.423	0.52	0.00071
X	0.583	0.687	0.583	0.685	0.013

Table 3. Hotspots for LOH in the epithelium (a) and the stroma (b).

(a)

Marker	number of informative cases in the epithelium	Observed LOH frequency in the epithelum	Model-based p-value for hotspot in the epithelium	Model-based p-value for hotspot in the stroma
D6S1277	26	1	0	0.0018
D22S683	35	0.829	6.8E-07	0.55
D3S1763	45	0.8	1.7E-06	0.00014
D17S1294	56	0.804	4.2E-06	0.00076
D3S2427	56	0.786	4.7E-06	0.073
D12S395	65	0.754	5.2E-06	9.5E-07
D9S922	52	0.808	6.8E-06	0.061
D1S2134	46	0.761	1.0E-05	0.00025
D16S2621	43	0.767	1.5E-05	0.0015
D2S1334	28	0.929	1.7E-05	0.00034
D3S1746	55	0.727	2.7E-05	0.0013
D4S2417	39	0.821	3.5E-05	0.0016
D13S800	35	0.857	3.5E-05	0.00075
D12S2078	44	0.773	6.7E-05	0.00013
D10S2470	53	0.792	0.00012	0.0011
D12S297	33	0.818	0.00013	0.0040

(b)

Marker Marker	number of informative cases in the stroma n.info.ST	Observed LOH frequency in the stroma p.LOH.ST	Model-based p-value for hotspot in the stroma gee1.ST.p	Model-based p-value for hotspot in the epithelium gee1.EP.p
D12S395	60	0.817	9.5E-07	5.2E-06
D14S606	40	0.85	5.7E-06	0.00019
D20S481	59	0.78	9.6E-06	0.0054
D16S753	72	0.778	1.3E-05	0.027
D2S434	44	0.795	6.2E-05	0.0034
D3S1764	54	0.796	0.00012	0.14
D1S3721	68	0.735	0.00012	0.0036
D12S2078	36	0.806	0.00013	6.7E-05

Table 4. Previously found hotspots in sporadic breast cancer [**For Supplement Only**]

marker	Whether the marker is a hotspot in the epithelium (EP) or stroma (ST)	Model-based p-value for hotspot in prostate cancer study
D1S549	EP	0.22
D2S1790	EP	0.0042
D2S1776	EP	0.00049
D3S2398	EP	0.0023
D4S2368	EP	0.026
D5S1505	EP	0.00020
D9S930	EP	0.0067
D10S1423	EP	0.017
D11S2002	EP	0.14
D11S1986	EP	0.013
D12S297	EP	0.00013
D13S894	EP	0.0035
D13S793	EP	0.051
D15S822	EP	0.30
D17S1298	EP	0.025
D17S974	EP	0.016
GATA178F11	EP	0.0084
D19S714	EP	0.060
D20S480	EP	0.0012
D1S2134	ST	0.00025
D2S1790	ST	0.0067
D2S1334	ST	0.00034
D2S1776	ST	0.11
D3S1746	ST	0.0013
D3S2398	ST	0.0017
D4S2368	ST	0.088
D4S2417	ST	0.0016
D6S1959	ST	0.16
D6S1056	ST	0.004
D8S1136	ST	0.00073
D10S1423	ST	0.035
D10S1222	ST	0.084
D11S2365	ST	0.00078
D11S4459	ST	0.13
D11S2006	ST	0.0089
D11S4464	ST	0.00032
D12S2070	ST	0.97
D12S2078	ST	0.00013
D13S894	ST	0.27
D13S800	ST	0.00075
D13S793	ST	0.25
D14S599	ST	0.84
D15S822	ST	0.41
D15S652	ST	0.031

D16S748	ST	0.0025
D16S753	ST	1.3E-05
GATA178F11	ST	0.029
D18S1364	ST	0.19
D19S1034	ST	0.035
D20S477	ST	0.13
D20S480	ST	0.00056
D21S1411	ST	0.0050
D22S686	ST	0.017
DXS1068	ST	1
DXS9893	ST	0.0085

Table 5. Chromosome-wise association between LOH and tumor grade.

Chromosome	p-value for association of LOH in the epithelium with tumor grade	p-value for association of LOH in the stroma with tumor grade
1	0.020*	0.024*
2	0.063	0.0051*
3	0.025*	0.068
4	0.0022**	0.00048**
5	9.8E-05**	0.01*
6	0.060	0.038*
7	0.040*	0.10
8	0.0015**	0.049*
9	0.19	0.0097*
10	0.021*	0.036*
11	0.43	0.087
12	0.090	0.023*
13	0.21	0.0066*
14	0.00050**	0.13
15	0.031*	0.0068*
16	0.00086**	0.0056*
17	0.37	0.013*
18	0.0021**	0.015*
19	0.069	0.011*
20	0.23	0.48
21	0.21	0.0083*
22	0.015*	0.030*
X	0.21	0.029*

*The p-values that are statistically significant at the 0.05 level
** p-values which remain significant after Bonferroni correction

Figure legends

Figure 1. Observed LOH frequencies (top panel) and model-based estimates for LOH frequencies (bottom panel) in the epithelium and the stroma. Each chromosome is labeled with its name, and the diagonal line representing equal LOH frequency in the two compartments is drawn in each plot for comparison. In the bottom panel, chromosomes for which the comparison between two frequencies is significant at the 0.05/23 level are marked in red.

Figure 2. Multi-dimensional scaling of all 232 samples as an illustration of whether the LOH/AI pattern of epithelium and stroma originating from any single tumor is more similar than the LOH/AI pattern amongst all epithelium samples and all stromal samples. The distance between two points is approximately the proportion of discordant LOH between the two corresponding samples. The epithelium (red dots) and stroma (blue dots) samples from the same subject are connected by a line segment. Therefore, short line segments indicate relative similarity between epithelium and stroma samples from the same subjects.

Figure 3. Distribution of percentages of discordant LOH between paired epithelium and stroma samples (top panel), and those between all other pairs (bottom panel).

[**For Supplement Only**]Figure 4. Hierarchical clustering of the 116 subjects with epithelium and stroma sample combined. For each sample, the sample number followed by information for all three CPF (lymph node metastasis, tumor grade and tumor size) is labeled. The following notations were used. lymph node metastasis (LN): 0=no, 1=yes, 9=N/A; tumor grade (Gd): 1=N/A; tumor size (Sz): in cent-meters.

[**For Supplement Only**]Figure 5. Hierarchical clustering of the 116 samples based on hotspots. For each sample, the sample number and information for all three CPF are labeled in the same way as for Figure 4.

Figure 6. Distributions of predicted probabilities of having a high (at least 7) grade tumor based on a logistic regression model for subjects with tumor grade 5, 6, 7 and 8. The mean of each distribution is marked with a red vertical line.

Competing interests statement

The authors declare that they have no competing financial interests